A COMPARISON OF THE EFFECTS OF CYANIDE AND AZIDE ON THE DEVELOPMENT OF FROGS' EGGS [1]

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Loeb's (1895) observations that the eggs of *Fundulus heteroclitus* are capable of considerable development under anaerobic conditions has since been extended to various amphibian embryos. Brachet (1934), in confirming the possibility of anaerobic development for *Rana temporaria* eggs, reported also that cyanide is similar to anaerobiosis in its effects on embryogenesis. Eggs placed in cyanide immediately after fertilization were arrested in the late blastula, but those placed in cyanide after gastrulation had begun would continue to the formation of a complete blastopore. Later stages were increasingly sensitive to cyanide. Although it has generally been thought that the arrests of development caused by cyanide are due to inhibition of the cytochrome oxidase of the Warburg-Keilin system (Keilin, 1933), it might be inferred from the recent work of Holtfreter (1943) that the repressive effects of cyanide solutions result merely from their alkalinity. It will be shown in this paper however that only post-mortem effects are influenced by the pH of the cyanide solution, the actual stoppage resulting from the presence of the toxic radical itself.

In 1936 Keilin reported in detail on another specific inhibitor of cytochrome oxidase, sodium azide (NaN_3). On the basis of these experiments NaN_3 and NaCN have in some cases been used interchangeably. Philips (1940), in comparing the developmental sensitivities to anaerobiosis of pelagic and non-pelagic fish eggs, employed both NaCN and NaN_3. He found that Fundulus eggs before the end of gastrulation are capable of extensive development in concentrations of both cyanide and azide which completely and almost immediately inhibit pelagic eggs. Except for the higher concentrations required in the case of NaN_3 he could demonstrate no difference between the effects of the two reagents. Recently Barnes (1944) tested the same reagents on the development of *Rana pipiens*. The results with cyanide confirmed the earlier observations of Brachet (1934). While no detailed data are given, the effects of azide were apparently found to completely parallel those of cyanide, for Barnes states: "Eggs exposed to M/100 NaN_3 at pH 7.0 are able to develop to the gastrula stage. Gastrulation never occurs in the presence of azide." Lower concentrations (M/1000) did not stop gastrulation although the eggs developed at a slower rate.

The present authors (Moog and Spiegelman, 1942), while investigating the relation between regeneration and metabolic activity, demonstrated a specific difference between the effects of azide and cyanide on hydranth reconstitution in Tubularia. Azide could inhibit regeneration at concentrations which did not sensibly

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affect respiration whereas cyanide caused parallel depressions in rates of regeneration and of respiration. Subsequent analysis (Spiegelman and Moog, 1944) of the differential effects of these two agents on the mass and time of appearance of the new hydranth emphasized the difference in their activities.

In the fall of 1941 the authors undertook a comparison of the effects of NaCN and NaN_3 on the development of *Rana pipiens*.[3] The results obtained disagree in certain respects with those reported by Barnes (1944). Azide was found to be completely effective in stopping morphogenesis at all stages of development, including those between fertilization and gastrulation which are not inhibited by cyanide. In an effort to discover the cause for the disagreement these experiments were repeated recently under conditions closer to those employed by Barnes. Our earlier results were confirmed and the discrepancy remains unresolved. No direct comparison with the findings of Philips (1943) is possible, not only because of the difference in material but also because the highest concentration he employed was below the one we found to give consistent inhibitions.

The results will be detailed and the difference obtained between the effects of azide and cyanide will be discussed in the light of recent findings on azide inhibitions of anaerobic synthetic processes.

General Methods and Materials

Eggs of *Rana pipiens,* obtained by injection of pituitary glands, were expressed and artificially fertilized. After swelling of the jelly the eggs were cut up into small groups in 10 per cent Ringers solution adjusted to the desired pH with phosphate buffer. Stages were determined according to the schedule of Pollister and Moore (1937) and are so numbered in the present paper. The eggs were stripped from the jelly with fine forceps before being immersed in the experimental solutions.

All hydrogen ion concentrations were determined with a glass electrode after the reagents were added. Where temperature control is indicated the designated temperature was held within $\pm$ 0.2° C. Other experimental details will be found in the appropriate places of the text.

Experimental Results

The effects of azide and cyanide on development

Keilin (1936) as well as subsequent investigators demonstrated the critical influence of pH on the effectiveness of azide as a respiratory inhibitor. Using the isolated Warburg-Keilin system as well as yeast cells Keilin obtained maximal effects at about pH 6.3 when the azide was used in concentrations of 0.001 and 0.002 molar. In the experiments to be described in the present section azide solutions were adjusted to pH 6.6. The concentration chosen for study was 0.005 molar, since parallel experiments on the effects on respiration (see Spiegelman and Steinbach, 1945) indicated maximal effects at this concentration on respiratory rate. The same can be said for development, for 0.005 M azide yields completely effective inhibition. All controls for the azide experiments were similarly adjusted

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to pH 6.6. In the case of cyanide both experimentals and controls were run at pH 8.4. The controls at pH 8.4 did not differ detectably in rate of development from those at pH 6.6. Every experimental set had its own control and both were thus handled exactly the same number of times and in the same fashion. This avoided the relatively more frequent handling and examination of the controls which would have been necessary if one set of eggs were the controls for a larger number of experimentals. For convenience in observations all of the present experiments were done at 15.2° C. in a cold room. To avoid the accelerating and decelerating effects of changing temperatures during development (see Ryan, 1943) the eggs were kept at 15.2° C. in 10 per cent Ringers until they reached the stage it was desired to test. They were then transferred to the approximate solutions previously brought to the same temperature. The cyanide solutions were freshly prepared and renewed every 12 hours during the course of an experiment; the experimental solutions were kept in stacked fingerbowls, with an empty bowl covering the top member of the stack.

For the purposes of comparison with the azide experiments, the results with cyanide in the early stages are reproduced in Table I. They do not differ in essentials from those reported by Brachet (1934). Eggs placed in cyanide early in development, although delayed as compared with controls, continue to develop up to gastrulation. The later the stage at which they are subjected to cyanide the closer is the approach to gastrulation; they do not however actually begin to gastrulate. Eggs in early stage 9 will continue to segment until the cells at the vegetal pole are quite minute but will evidence no signs of dorsal blastopore lip formation. If however the invagination has already started cyanide will not immediately stop it and the eggs may proceed to the formation of a complete blastopore before ceasing activity. Later stages become increasingly sensitive to cyanide.

TABLE I

The effects of cyanide on development at pH 8.4 at 15.2° C. These experiments were done in 1941–2 on material obtained from Vermont. The numbers represent the developmental stages as described under Methods.

Stage at start of experiment	Solution	Hours after immersion											No. of eggs
		4	8	12	18	24	35	45	55	75	95	120	
Uncleaved	0.001 M	2	3		6	8	8	8	8				210
Uncleaved	Control	3	3		8	8	10	11	12				210
3	0.001		4		6	7	8	8	9	9	9		200
3	Control		6		8	9	10	11	12		16		200
6	0.001		7			8	8	8	8	9	9	9	160
6	Control		8			10	11	12	12	13	16	18	160
9	0.001		9		9		9	9					200
9	Control		10		11		12	13		16			200
10	0.001			10		10		11		12		12	200
10	Control			11		12		14		16		18	200

The results obtained with azide are summarized in Table II. It is immediately evident that all stages are azide sensitive, including the early ones which are not effectively inhibited by cyanide. It might be noted that under these experimental conditions the cessation of developmental activity on immersion in azide solution is, as far as can be determined, abrupt and immediate. This was easily ascertained

TABLE II

The effect of azide on development at pH 6.6 at 15.2° C.; 1941–2, material from Vermont. The numbers represent the developmental stages as described under Methods

Stage at beginning	Solution	Hours after immersion											No. of eggs
		4	8	12	18	24	35	45	55	75	95	120	
Uncleaved	0.005 M	1		1			1	1	1	1	1	1	100
Uncleaved	Control	3		5			10	11	12	14	16	18	100
6	0.005 M		6			6	6	6	6	6	6	6	150
6	Control		7			9	10	11	12	14	16	18	140
7	0.005 M		7	7		7		7	7	7	7	7	90
7	Control		9	10		11		12	14	16	17	18	90
9	0.005 M	9			9			9		9	9	9	110
9	Control	10			11			13		16	17	18	110
10	0.005 M			10			10			10	10		160
10	Control			11			13			17	18		160
11	0.005 M		11				11		11		11		85
11	Control		12				14		16		18		85
12	0.005 M					12			12				105
12	Control					14			16				105
13	0.005 M		13			13	13		13				120
13	Control		14			16	17		18				120
14	0.005 M				14		14	14			14		90
14	Control				16		17	18			19		90
16	0.005 M			16			16		16	16			110
16	Control			17			18		18	19			110
17	0.005				17	17	17	17	17				60
17	Control				18	18	18	18	19				60

in the early cleavage stages since no further cleavage was observed. Although the observations are more difficult in the later stages, careful examination failed to reveal any development subsequent to treatment with azide. If the eggs are removed within 30 minutes after being placed in the azide solution and thoroughly washed they can proceed with their development.

Barnes' (1944) experiments with azide were done at higher temperatures, concentrations, and pH than those described above. Accordingly when the azide ex-

periments were repeated they were done at room temperature (ca. 25° C.), at pH 7.4 and 8.3 (i.e. with and without added hydrochloric acid), and with concentrations up to 0.01 M. The results of these experiments are given in Table III. At both hydrogen ion concentrations, 0.01 M azide caused immediate arrest in all pregastrular stages. The 0.005 M concentration used in the early experiments was retested under these conditions and found to give exactly the same results as previously obtained. Controls kept in Ringers buffered at the experimental pH developed normally in all cases, and are not reported in the table.

TABLE III

The effect of azide on development at 25° C.; 1944–5, material from Wisconsin

Stage at immersion	Conc. (Molar)	pH	Stage at arrest
1	0.001	7.4	9*
1	0.001	8.3	9*
1	0.005	7.4	1
1	0.005	8.3	1
1	0.01	7.4	1
1	0.01	7.4	1
1	0.01	8.3	1
7	0.005	7.4	7
7	0.01	7.4	7
9	0.005	7.4	9
9	0.005	7.4	9
11+	0.01	8.3	12−
13	0.01	8.3	13

* There was no delay in reaching this stage.

It is clear that we can offer no support to Barnes' statement that at the concentration and pH she employed, azide, like cyanide, permits eggs to develop to gastrulation.

The effect of NaCN on development at different pH values

Holtfreter (1943) presented evidence showing that the disruptive effects of strong cyanide solutions (0.1 M to 0.0015 M) can be imitated by potassium hydroxide solutions of equal pH. Although the author did not specifically claim that the oxidation-repressing effects of cyanide are to be regarded as completely irrelevant to its influence on development, it nevertheless seemed advisable to us to clarify the points which were left in an indecisive state by Holtfreter's work. This we did, in our 1944–1945 series of experiments, both by comparing the effects of NaCN solutions brought to pH 7.2 with HCl with those at pH 9.6–9.8, and by determining the effects of solutions of either NaOH or KOH at pH 9.8. The tests were made at about 25°; the cyanide solutions were changed three times daily, the hydroxide solutions once daily.

The results of the NaCN tests completely confirmed our earlier findings (Table IV). The stage in which development was stopped, and the speed with which that stage was reached, was in all cases the same in solutions of equal concentration at both low and high pH. Only after the egg had been in an arrested stage for

more than 12 hours did a difference between the two pH's become evident. At high alkalinity the pigment became streaked, the surface disintegrated, and the egg was in the majority of cases reduced to a loose, fuzzy mass of cells within 36 hours; at low alkalinity the surface was only moderately eroded after 72 hours.

TABLE IV

The effects of NaCN at pH 7.2 and 9.8; 1944–5, material from Wisconsin

Stage at immersion	Conc. (Molar)	pH 7.2		pH 9.6–9.8	
		Stage at arrest	Later effects	Stage at arrest	Later effects
1	0.003	Not tested		7	Egg swollen and surface severely depigmented after 36 hrs.
1	0.006	Not tested		7	
7	0.003	9	Marked depigmentation after 40 hrs.	9	Depigmentation after 20 hrs., surface disintegrated after 36 hrs.
7	0.006	9		9	
9	0.003	11	Blastopore lip disappeared within 20 hrs. after forming	11	Blastopore lip also disappeared. Surface completely disintegrated after 24 hrs.
9	0.006	11		11	
1	0.004	7	Surface became mottled but did not disintegrate within 72 hrs.	7	Complete disintegration within 24 hrs.
1	0.004	7	Egg swelled to twice its normal diameter but did not disintegrate within 96 hrs.	7	Complete disintegration within 24 hrs.
8	0.004	9	Surface became mottled and egg swelled somewhat, but did not disintegrate within 88 hrs.	9	Surface became mottled within 24 hrs., complete disintegration within 38 hrs.

The studies with hydroxides revealed that *Rana pipiens* eggs can develop from fertilization to the stage of tail-fin circulation (stage 22, at which they were discarded) at pH 9.8 (i.e., 2.5×10^{-4} M). Stage 22 was also achieved uneventfully if the eggs were immersed in the hydroxide solutions at the stage of the morula (S7), late blastula (S9), mid-gastrula (S11), neurula (S14), muscular movement (S18); in the last two cases the vitelline membrane was removed before the embryos were placed in the alkali solutions. In complete contradiction to Holtfreter's finding that eggs disintegrate in the morula stage in KOH solutions of pH 9.0 to 9.4, we did not observe either retardation or abnormality of development. In three experiments with NaOH and two with KOH, we obtained identical results. Thus we may conclude that the suppressive action of NaCN (or KCN) on living egg is due to the poisonous effect of the CN component.

Discussion

Both azide and cyanide are effective inhibitors of respiration in the early as well as in the later stages of development (Barnes, 1944; Spiegelman and Steinbach, 1945). The fact that cyanide cannot inhibit at any stage before gastrulation whereas azide can inhibit at all stages, cannot be explained on a respiratory basis. This is even more pointedly demonstrated by the capacity of eggs to develop to gastrulation under anaerobic conditions. The ability of cyanide to depress respiratory rates at all stages clearly proves that it gets into the cells of the early embryos, and consequently a difference in permeability cannot be invoked to explain the difference between the effects of azide and cyanide on development. It is clear from these experiments that, at least in the early stages, NaN_3 is inhibiting some cyanide-insensitive process necessary for development.

Recent work has served to question conclusions drawn from Keilin's earlier experiments that azide and cyanide are essentially equivalent inhibitors of the Warburg-Keilin system. Stannard (1939) showed that cyanide inhibited the respiration of both resting and active muscle while azide affected active muscle only. Armstrong and Fisher (1940) demonstrated that azide and cyanide behave differently in inhibiting the enzymes controlling the frequency of the embryonic fish heart-beat. Differences in cyanide and azide inhibitions of tissue respiration led Korr (1941) to postulate the existence of different pathways of respiration in resting and stimulated tissues. Ball (1942) suggested different oxidation-reduction potentials for the Atmungsferment-cyanide and Atmungsferment-azide compounds as an explanation of the different effects of the two inhibitors. Winzler (1943), after subjecting the kinetics of the respiratory inhibition by cyanide and azide in yeast to a careful examination, came to the conclusion that cyanide inhibited yeast respiration by three different pathways: (1) by combining with oxidized Atmungsferment; (2) by increasing the apparent KO_2 of reduced Atmungsferment; and finally (3) by combining with the enzyme which controls the rate-limiting step of the rate of respiration. Azide on the other hand exhibited only one type of inhibition, namely, combination with oxidized Atmungsferment.

Aside from these studies on respiration, others have been made on assimilatory activity of microorganisms. Barker (1936) and Giesberger (1936) showed that suspensions of bacteria could under certain circumstances synthesize carbohydrate from various substrates. Clifton (1937) studied the effect of azide on these syntheses and found them to be completely inhibited. In the presence of azide external substrate was completely oxidized. Clifton and Logan (1939) extended these findings and showed that it was possible to differentially inhibit assimilatory processes with both NaN_3 and 2, 4-dinitrophenol. Winzler (1940), working with acetate assimilation in yeast, showed that low concentrations of azide, cyanide, or 2, 4-dinitrophenol prevented assimilation. Azide was also shown by Winzler (1944) to prevent the anaerobic assimilation of glucose by yeast without interfering with its fermentation. Winzler, Burk, and du Vigneaud (1944) found that azide in concentrations of 10^{-4} and 10^{-3} molar inhibits completely the anaerobic assimilation of ammonia.

These experiments show that azide, and in certain instances cyanide, can inhibit synthetic processes which are essentially anaerobic in nature and not connected with the Warburg-Keilin system. It seems most probable that it is this

sort of inhibitory activity which is involved in the ability of azide to stop embryonic development. Unfortunately, with the exception of Winzler's (1940) study of acetate assimilation no detailed comparison between the effects of azide and cyanide on synthetic processes has been published. In view of the results reported in the present paper one would venture to predict that such differences will be discovered. It may be noted that one such difference has been found in the case of adaptive enzyme formation in yeast, which is azide sensitive but is not inhibited by cyanide (Spiegelman, 1945). A suggestive finding has been reported recently by Meyerhof (1945), who prepared a solution of adenylpyrophosphatase from yeast by supersonic vibration and found it insensitive to cyanide but highly sensitive to azide. This enzyme, involved as it is in transphosphorylation, might conceivably be a part of the azide sensitive anaerobic synthetic processes.

Summary

Previous observations that amphibian eggs can develop up to the beginning of gastrulation in cyanide solutions have been confirmed on eggs of *Rana pipiens*. The effect of cyanide is independent of pH, and eggs can develop into tadpoles in 2.5×10^{-4} molar NaOH or KOH solutions at pH 9.8.

Azide has been found to arrest development immediately in all stages from fertilization to tail-bud formation. The effect is the same from pH 6.6 to pH 8.3.

These differences are discussed in the light of recent studies on the effects of azide and cyanide on respiratory, assimilatory, and phosphorylative processes.

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